METHOD

The Analysis of Lipids *via* HPLC with a Charged Aerosol Detector

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ABSTRACT: Because most lipid extracts are a mixture of saturated and unsaturated molecules, the most successful strategies for the quantitative analysis of lipids have involved the use of so-called "mass" or universal detectors such as flame ionization detectors and evaporative light scattering detectors. Recently a new type of HPLC "mass" detector, a charged aerosol detector (CAD), was developed and is now commercially available. This detection method involves nebulizing the HPLC column effluent, evaporating the solvents, charging the aerosol particles, and measuring the current from the charged aerosol flux. In the present study, the CAD was evaluated with several normal phase and reverse phase HPLC methods commonly used for the quantitative analysis of lipid classes and lipid molecular species. The CAD detected common lipids such as triacylglycerols, diacylglycerols, glycolipids, phospholipids, and sterols. Lower molecular weight lipids such as free FA had smaller peak areas (50–80% lower). FAME were not detected by the CAD, probably because they were completely evaporated and did not form aerosol particles. The minimum limits of detection of the CAD with lipids varied with different mobile phase solvents. Using solvent systems that were predominantly hexane, the minimum limits of detection of triacylglycerols, cholesterol esters, and free sterols were about 1 ng per injection and the mass-to-peak area ratio was nearly linear from the range of about 1 ng to about 20 mg per injection. Three other solvents commonly used for HPLC lipid analysis (methanol, isopropanol, and acetonitrile) caused higher levels of background noise and higher minimum limits of detection. These experiments indicate that the CAD has the potential to become a valuable tool for the quantitative HPLC analysis of lipids. Long-term studies are needed to evaluate full instrument performance.

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Several reviews have described in detail the numerous HPLC methods that have been developed for the quantitative analysis of plant, animal, and microbial lipids (1–4). The most common and least expensive detection method for HPLC is the UV-visible detector (1). UV-visible detectors have proven to be useful for those lipids that have chromophores, but they have been of limited use for the analysis of lipid extracts that contain a mixture of saturated and unsaturated molecules. Beginning in the mid-1980s, the first HPLC methods were developed for the quantitative analysis of lipids using "mass" detectors such as FID and ELSD, which could detect both saturated and unsaturated lipids. The first method that employed an ELSD for the analysis of lipid classes was published by W.W. Christie in 1985 (5). In 1990, our group published a similar HPLC method for the analysis of lipid classes using an FID (6) and demonstrated that FID and ELSD technologies were comparable. Although FIDs were successfully used for HPLC analysis for several years, FID technology was quickly surpassed by ELSD technology, and the manufacturing of FID ceased in the mid-1990s. In the last 15 years the sensitivity of ELSD has improved greatly and numerous methods have been developed for the analysis of lipids by HPLC-ELSD (1,3). Whereas the minimum limits of detection with early ELSD were about 10–20 μg per peak, the minimum limits of detection of lipids with modern ELSD has improved to about 50 to 100 ng (1).

Besides the FID and ELSD, a third type of aerosol-based detector was reported with examples of applications for lipid analysis (7). When used with a microbore column, this condensation nucleation light scattering detector (not commercially available) was reported to have minimum limits of detection of less than 1 ng.

Recently a new type of HPLC "mass" detector, a charged aerosol detector (CAD), was developed and is now commercially available (8,9). This detection method involves nebulizing the HPLC column effluent, evaporating the solvents, charging the aerosol particles, and measuring the current from the charged aerosol flux. In the present study, the CAD was evaluated with several normal phase and reverse phase HPLC systems commonly used for the quantitative analysis of lipid classes and lipid molecular species.

EXPERIMENTAL PROCEDURES

Materials. All organic solvents were freshly opened bottles of Baker Analyzed® HPLC grade solvents, obtained from Mallinckrodt Baker Inc. (Phillipsburg, NJ). Chromatographic standards of lipids [Non-polar Lipid Mix A (cat# 1129), Non-polar Lipid Mix B (cat# 1130), and Polar Lipid Mix (cat# 1127), all for TLC] were obtained from Matreya (Pleasant Gap, PA). Lecithin granules (97% soy phosphatides) were obtained from the Vitamin Shoppe (North Bergen, NJ). Barley lipid extract was obtained by extracting ground barley kernels (the cultivar was Doyce) with hexane, as previously described (10).

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Abbreviations: CAD, charged aerosol detector; T, tocopherol; T3, tocotrienol.

HPLC system. The HPLC was an Agilent Model 1100 with autosampler. For experiments that involved injecting various masses of lipids, solutions were prepared at concentrations of 0.001, 0.01, 0.1, and 1.0 mg/mL, and triplicate injections of 1, 2, 5, and 10 μL of each concentration were made. Detection was by two methods, in series. An Agilent Model 1100 Fluorescence Detector (Agilent Technologies, Avondale, PA), with excitation at 294 nm and emission at 326 nm (flow cell volume, 8 μL) was used upstream of an ESA Corona™ Charged Aerosol Detector (ESA Biosciences, Chelmsford, MA) operated with nitrogen as a nebulizing gas and at a range of 500 pA. All other CAD parameters were preset by the manufacturer. The volume of solvent in the 50 cm of 0.010-in. i.d. PEEK tubing between the fluorescence detector and CAD was approximately 25 μL.

Normal phase nonpolar lipid HPLC analyses. This method was originally developed for use with an evaporative light scattering detector (11). A LiChrosorb 7 μm diol column (3 × 100 mm, packed by Chrompack, Raritan, NJ) was used. The binary gradient had a constant flow rate of 0.5 mL/min, with Solvent A = 99.9 % hexane/0.1% acetic acid (all solvent compositions are vol/vol), Solvent B = 99% hexane/1% isopropanol. Gradient timetable: at 0 min, 100/0 (%A/%B); at 8 min, 100:0; at 10 min, 75:25; at 40 min, 75:25; at 41 min, 100:0; and at 60 min, 100:0. These nonpolar lipid components were identified by comparison to the retention times of commercial standards.

Normal phase polar lipid HPLC analyses. Polar lipids (including acylated steryl glucosides, steryl glucosides, and phospholipids) were quantitatively analyzed by a similar method developed for use with an ELSD (12). The polar lipid components were identified by comparison to the retention times of commercial standards. The diol column and flow rates were the same as above. For isocratic analyses the mobile phase consisted of 45.9% hexane/50% isopropanol/4% water/0.1% acetic acid. For gradient analyses, the ternary gradient consisted of: Solvent A = 99.9% hexane/0.1% acetic acid, Solvent B = 100% isopropanol, and Solvent C = 100% water. Gradient timetable: at 0 min, 90:10/0 (%A/%B/%C); at 30 min, 58:40:2; at 40 min, 45:50:5; at 50 min, 45:50:5; at 51 min, 50:50:0; at 52 min, 90:10:0; and at 60 min, 90:10:0.

Normal phase tocopherol and tocotrienol HPLC analyses. Tocopherols and tocotrienols were quantified by using a modified version of the previously published method (13). The diol column and flow rates were the same as above. The binary gradient consisted of: Solvent A = 98% hexane/2% methyl *t*-butyl ether and Solvent B = 100% isopropanol. Gradient timetable: at 0 min, 100:0 (%A/%B); at 40 min, 100:0; at 45 min, 95:5; A/B, at 50 min, 95:5; at 51 min, 100:0; and at 60 min, 100:0. The MS was used to aid in peak identification. Gelcap supplements of tocopherols (Bio E Gamma Plex, Soloray Inc., Park City, UT) and tocotrienols (Tocopherol Complex, Solgar, Leonia, NJ) were purchased at a local vitamin store and the following peaks were confirmed by LC-MS, performed with an Agilent 1100 MSD equipped with an Atmospheric Pressure Chemical Ionization interface operated in the positive mode (drying gas at 6.0 L/min, nebulizer pressure at 60 psi, drying gas temperature at 350ºC, vaporizer gas temperature at 325ºC, capillary voltage at 4,000 V, and corona current at 4.0 μA, and fragmentor at 80 V): αT (M + 1 = *m/z* 431.4), αT3 (M + 1 = *m/z* 425.3), βT and γT (M + 1 = *m/z* 416.3), δT3 and γT3 (M + 1 = *m/z* 411.2), δT (M + 1 = *m/z* 402.3), and δT3 (M + 1 = *m/z* 397.1), where T = tocopherol and T3 = tocotrienol.

Reverse phase lipid molecular species HPLC analyses. This new method was recently developed to separate molecular species of triacylglycerols and other nonpolar lipids. The column was a Prevail RP18 3 μm (150 × 2.1 mm), packed by Alltech Associates, Deerfield, IL). The binary gradient had a constant flow rate of 0.5 mL/min, with Solvent A = 49.8% methanol/47% acetonitrile/3% dichloromethane/0.2% acetic acid, Solvent B = 100% isopropanol. Gradient timetable: at 0 min, 100:0 (%A/%B); at 20 min, 95:5; at 40 min, 50:50; at 50 min, 50:50; at 51 min, 100:0; and at 60 min, 100:0. These nonpolar lipid components were identified by comparison to the retention times of commercial standards.

RESULTS AND DISCUSSION

The first HPLC method to be evaluated with the CAD was a normal phase gradient elution method developed to quantitatively analyze the major nonpolar lipid class components (phytosterol esters, triacylglycerols, free FA, and free phytosterols) in vegetable oils and hexane extracts (11). The baseline was relatively smooth with this system (Fig. 1A). A commercial mixture that contained equal masses of five nonpolar lipid classes (cholesterol:oleate, triolein, oleic acid, methyl:oleate, and cholesterol) was injected in this system (Fig. 1B and C). Detector response (peak areas) for cholesterol:oleate and triolein were similar whereas that of cholesterol and oleic acid were approximately 10% and 80% lower, respectively. Methyl:oleate was not detected by the CAD, presumably because this lower MW component was completely evaporated and did not form aerosol particles. Similar results were previously reported for the ELSD—methyl esters were partially evaporated at a detector temperature of 40°C and completely evaporated at higher temperatures (1). Similarly (with the ELSD), free FA were partially evaporated at detector temperatures of 40°C and 60°C and completely evaporated at higher temperatures (1). Unlike most ELSDs, the nebulizer temperature of the CAD is preset and not variable. With this HPLC method, the minimum limits of detection with the CAD were about 1 ng, and the mass-to-peak area ratio was nearly linear from the range of about 1 ng to 20 ng per injection (Fig. 1D). In other experiments the standard curve was extended up to 20 μg and the mass-to-peak area ratio continued to remain nearly linear (data not shown).

The second HPLC method to be evaluated with the CAD was a normal phase method developed to quantitatively analyze the major polar lipid class components (mainly glycolipids and phospholipids) in extracts of plant material extracted with polar solvents (12). This system was evaluated with both isocratic elution (Fig. 2) and gradient elution (Fig. 3). In our

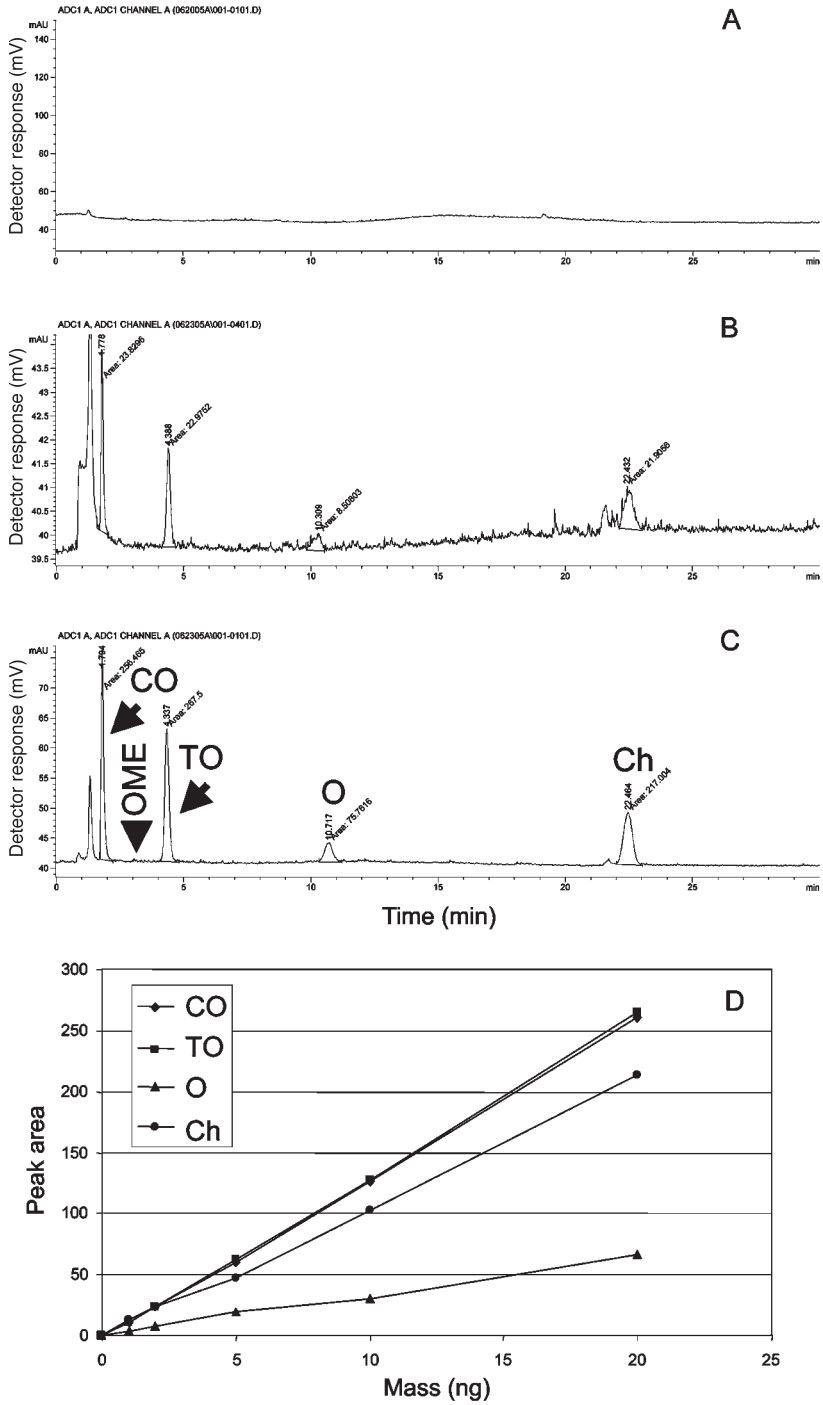

FIG. 1. Normal phase nonpolar lipid class HPLC analysis with detection via CAD. (A) Gradient blank, no lipid injected. (B) Matreya nonpolar lipid mix B with an injection comprised of 2 ng of each component. (C) Matreya nonpolar lipid mix B with an injection comprised of 20 ng of each component. (D) Mass versus peak area for Matreya nonpolar mix B. Abbreviations: CO, cholesteryl oleate; OME, oleate methyl ester; TO, triolein; O, oleic acid; Ch, cholesterol. Note that OME was totally evaporated and O was partially evaporated during detection and the other three lipids had similar detector response.

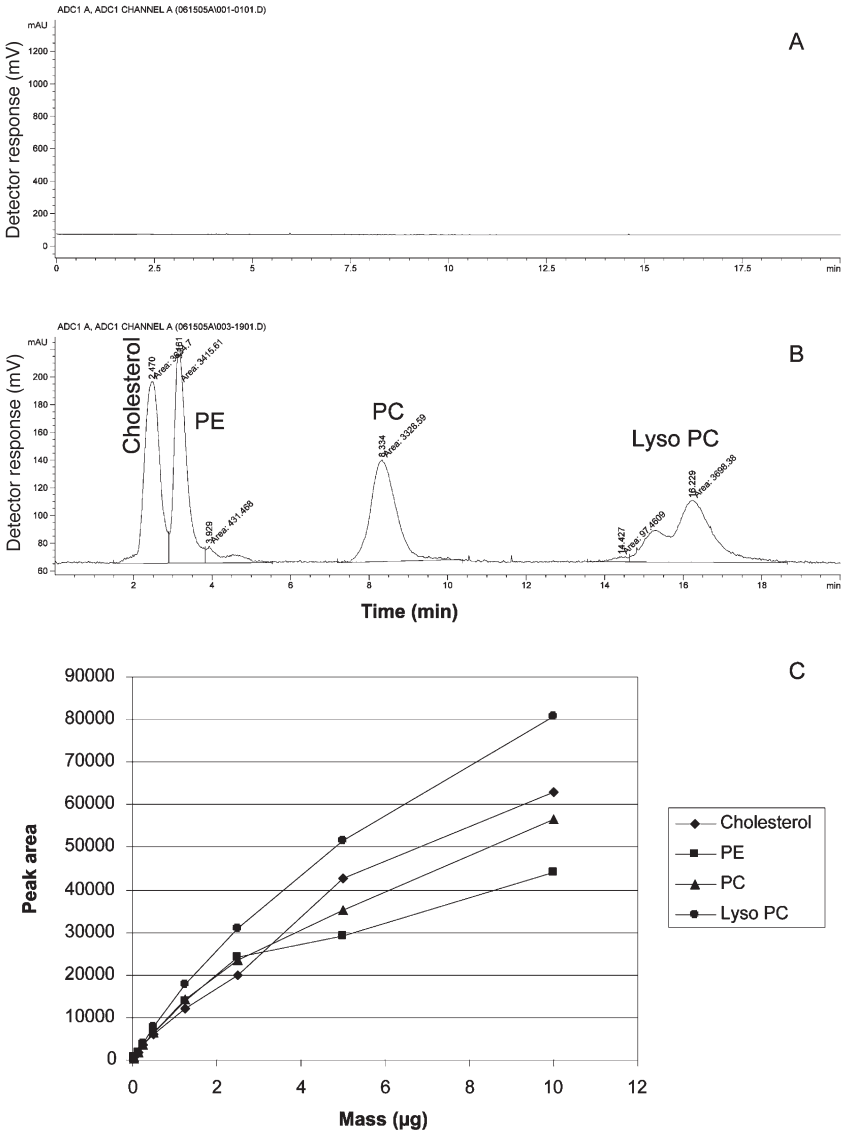

FIG. 2. Normal phase polar lipid class isocratic HPLC system. (A) Gradient blank, no lipid injected. (B) Matreya polar lipid mix with an injection comprised of 0.25 µg of each component. (C) Mass versus CAD peak area for Matreya polar lipid mix.

experience, the isocratic method is adequate to separate simple standard mixtures, but the gradient method is required to separate most plant lipid extracts. In the isocratic polar lipid system the baseline was relatively smooth (Fig. 2A). A commercial mixture that contained equal masses of four polar lipid classes (cholesterol, phosphatidylethanolamine [PE], phosphatidylcholine [PC], and lyso-phosphatidylcholine [lyso PC]) was injected in this system (Fig. 2B), and the components were well separated. With this HPLC system, the minimum limits of detection were about 25 ng, and the mass-to-peak area relationship was evaluated in the range from about 25 ng to about 10 µg per injection (Fig. 2C). Because it would have made the mass-to-peak area graph (Fig. 2C) difficult to read, the same data, with standard deviations are also reported in Table 1.

In using the normal phase polar lipid class gradient method, the baseline was relatively smooth until about 35 min, but it increased and "plateaued" from about 40 to 55 min, and then dropped back down to the original level for the remainder of the chromatogram (Fig. 3A). The four polar lipid classes in the standard were also well resolved in the gradient system (Fig. 3B). A sample of soy lecithin was then injected (Fig. 3C) and the CAD detected the same major glycolipids (acylated steryl glycoside and steryl glucoside) and phospholipids (PE, PC, lyso PC, and PI) that we previously observed by using an evaporative light scattering detector (14).

The next HPLC method evaluated was a normal phase method developed to separate the eight natural isomers of tocopherols and tocotrienols (13). Because tocopherols and to-

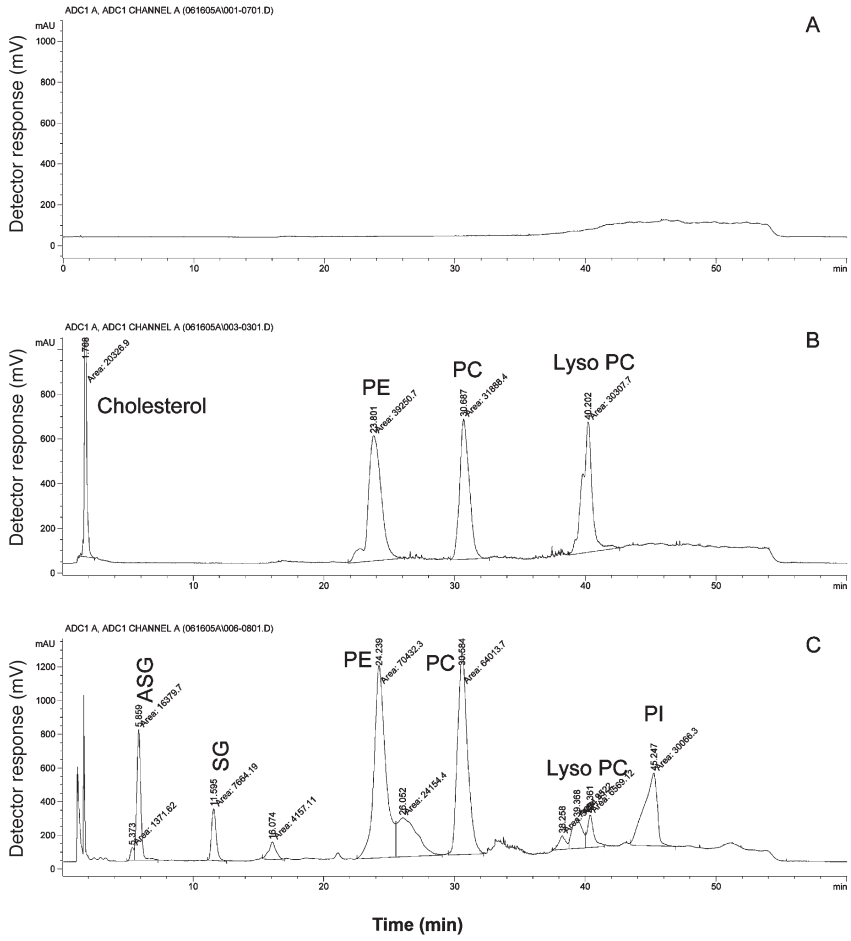

FIG. 3. Normal phase gradient chromatogram showing the separation and detector response of polar lipid standards. (A) Gradient blank, no lipid injected. (B) Matreya polar lipid mix with an injection comprised of 2.5 μg of each component. (C) Sample of soy lecithin, 20 μg of total lipid injected. Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; Lyso PC, lysophosphatidylcholine, ASG, acylated steryl glycoside; SG, steryl glucoside; PI, phosphatidylinositol.

TABLE 1
Response of CAD with Polar Lipid Standards with the Isocratic HPLC Method with a Diol Column and a Mobile Phase of 45.45% Hexane/50.00% Isopropanol/4.50% Water/0.05% Acetic Acid by Volume[a]

Injected (μg)	Cholesterol (peak area ± SD)	PE (peak area ± SD)	PC (peak area ± SD)	Lyso PC (peak area ± SD)
0.025	735 ± 311	397 ± 74	408 ± 65	438 ± 108
0.05	669 ± 65	724 ± 48	756 ± 37	644 ± 158
0.125	1,753 ± 95	1,804 ± 53	1,722 ± 90	1,880 ± 129
0.250	3,647 ± 29	3,410 ± 31	3,381 ± 100	3,762 ± 182
0.5	5,986 ± 347	6,375 ± 126	6,418 ± 151	7,786 ± 261
1.25	12,070 ± 174	13,820 ± 37	14,120 ± 449	17,620 ± 254
2.5	19,920 ± 268	24,160 ± 194	23,420 ± 428	30,790 ± 334
5.0	42,750 ± 490	29,120 ± 409	35,370 ± 682	51,570 ± 1482
10.0	63,000 ± 184	44,040 ± 1980	56,480 ± 2247	80,920 ± 3285

[a]The values for mean and SD were from three injections of each sample.

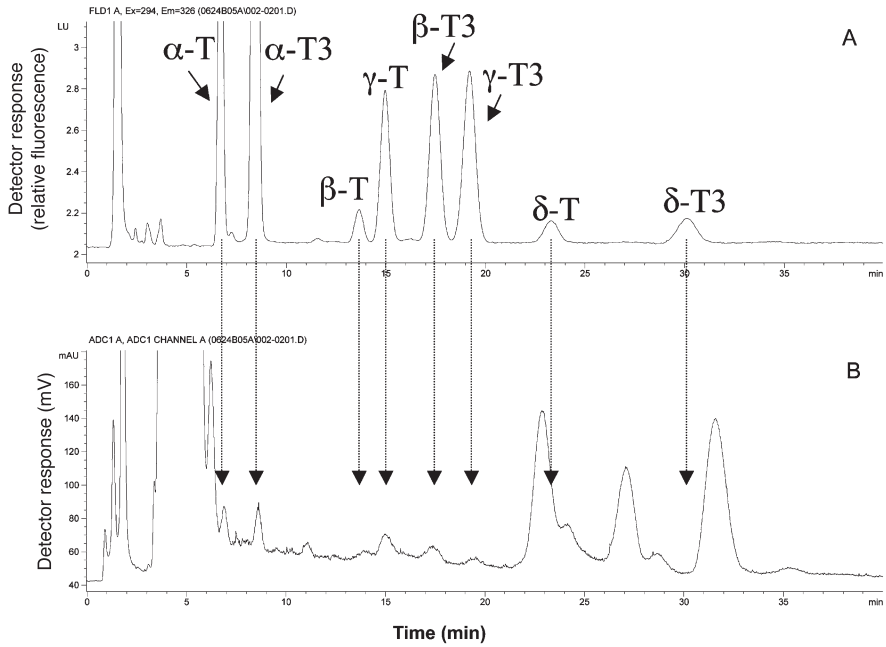

FIG. 4. Normal phase tocopherol/tocotrienol HPLC system showing the peaks of tocopherols and tocotrienols in unrefined oil obtained by extracting ground Doyce hulless barley kernels with hexane. (A) Detection was with a fluorescence detector (294 nm excitation and 326 nm emission). Abbreviations: T, tocopherol; T3, tocotrienol. (B) Detection with CAD.

cotrienols fluoresce, fluorescence detection (294 nm excitation and 326 nm emission) is a very selective detection method that has been used to accurately quantify these compounds, with minimum limits of detection of about 1 ng (15). Because the CAD also potentially has minimum limits of detection at about 1 ng, we prepared an extract of barley and compared the detection with the fluorescence detector (Fig. 4A) and the CAD (Fig. 4B). Interestingly, the CAD revealed several large peaks with retention times similar to tocopherols and tocotrienols (but since the components in these peaks did not fluoresce, they were not tocopherols or tocotrienols). Although it appears that the CAD could potentially be used to quantify some tocopherols and tocotrienols, the presence of other unknown peaks with similar retention times complicates the chromatogram. Under these conditions, it appears the fluorescence detection is still the HPLC detection method of choice for the quantitative analysis of tocopherols and tocotrienols.

The final HPLC method studied was a reverse phase method recently developed to separate the molecular species of triacylglycerols (e.g., triolein and trilinolein) and other nonpolar lipids. The baseline was noisier with this reverse phase system than with the normal phase systems (Fig. 5A). The reason for this increased noise was not examined. The nonpolar lipids in two commercial standard kits were separated with this system, at component concentrations of 25 ng (Fig. 5B) and 200 ng (Fig. 5C). Because the system was quite noisy, the minimum limits of detection with this system appear to be about 25 ng.

While conducting the previous experiments it was observed that the CAD baseline was relatively smooth with some solvents and noisy with others. The next experiment was designed to measure the effect of various common HPLC solvents on the CAD baseline (Fig. 6) at a constant flow rate of 0.5 mL/min, without a column and without injecting any lipid samples. The HPLC pump was programmed to deliver hexane for 10 min and then perform a 1-min gradient to transition to 100% isopropanol for the next 9 min, and similarly transition to methanol, water, isopropanol, and finally return to hexane. Among the four solvents evaluated in the first experiment, methanol produced the highest CAD background (Fig. 6A). Among the four solvents evaluated in the second experiment, acetonitrile produced the highest CAD background (Fig. 6B). Methanol and acetonitrile are the most common HPLC solvents for reverse phase HPLC. Clearly, more studies are needed to fully investigate the effects of these solvents on the noise and performance of the CAD.

These preliminary results indicate that the CAD has the potential to become a valuable tool for the quantitative HPLC analysis of lipids. The major advantages of the CAD are its low minimum limits of detection and its nearly linear mass-to-peak area relationship for many types of lipids. Long-term studies are needed to confirm that the results are reproducible and that the instrument is durable and reliable.

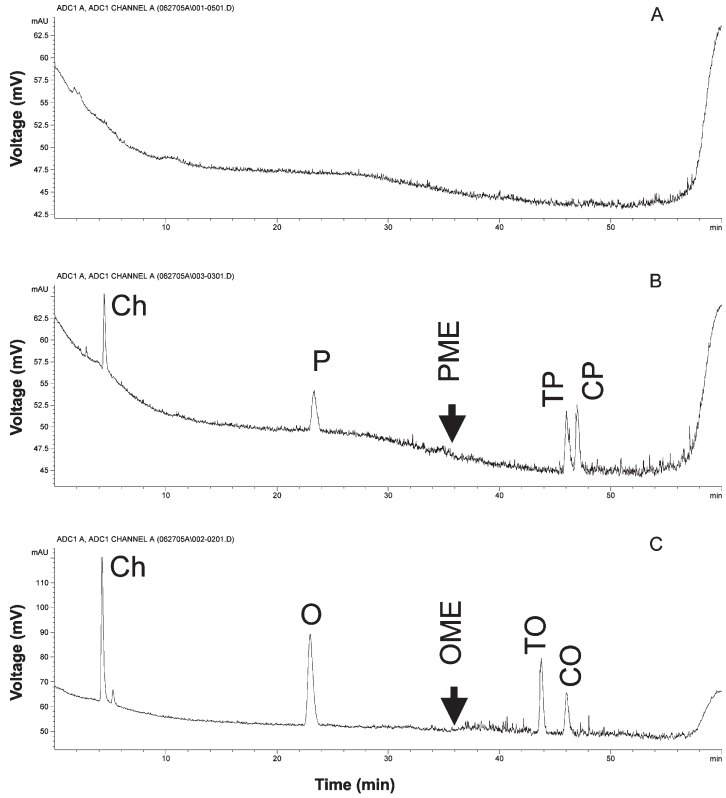

FIG. 5. Reverse phase nonpolar lipid HPLC system evaluated with Matreya nonpolar mix A and B. (A) Gradient blank, no lipid injected. (B) Matreya nonpolar lipid mix A with an injection comprised of 25 ng of each component. (C) Matreya nonpolar lipid mix B with an injection comprised of 200 ng of each component. Abbreviations: CP, cholesteryl palmitate; PME, palmitate methyl ester; TP, tripalmitin; P, palmitic acid; Ch, cholesterol. For other abbreviations, see Figure 1.

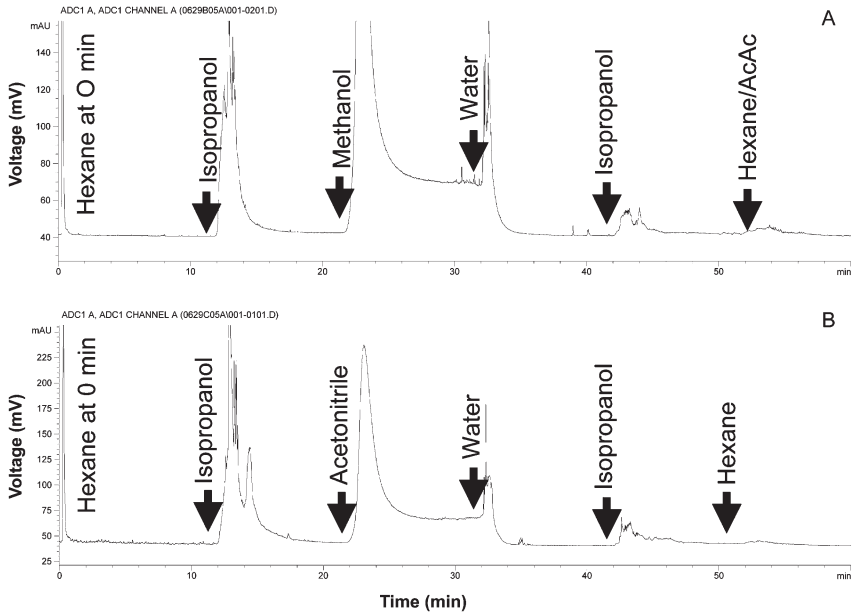

FIG. 6. The effect of various solvents on the CAD response with no HPLC column and no lipids injected. (A) Effect of hexane, isopropanol, methanol, and water (at a flow rate of 0.5 mL/min). (B) Effect of hexane, isopropanol, acetonitrile, and water (at a flow rate of 0.5 mL/min).

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